Original article:

Seroprevalence, clinical profile and knowledge, attitudes, and practices (KAPs) of brucellosis in North India in patients with pyrexia of unknown origin and chronic joint pain

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Abstract:

Introduction: Brucellosis is a significant but lesser-known cause of pyrexia of unknown origin (PUO) in India. Studies documenting the prevalence of Brucellosis in different parts of India are sparse and few. Clinicians thus usually don't consider it in their differential diagnosis. This study assesses its prevalence in two groups: febrile patients with unknown etiology and individuals presenting with arthritis and/or joint pains. Knowledge, Attitude, and Practices (KAP) among clinicians about the disease was evaluated. ***Materials and Methods:*** This is a prospective study conducted at a tertiary care center of North India catering to urban, semi-urban, and rural populace. Ninety-two patients with fever of unknown origin, arthralgia, or similar constitutional symptoms were recruited in this study. Detailed clinical history was elicited from all patients as per predesigned proforma and a rigorous physical examination was conducted. Following primary screening to rule out malaria, enteric fever, and leptospirosis, secondary screening for Brucellosis was done by Rapid Screen Test (PUO screen) and IgM and IgG ELISA. A predesigned survey was used for assessing KAP among clinicians about Brucellosis. ***Results:*** *Brucella* infection was diagnosed in 27 (29.3%) cases. The most common symptoms among the patients apart from fever were arthralgia (77.8%), fatigue (70.8%), pallor (66.1%), headache (59.2%), backache (53.8%) and cough (33.3%). PUO screen is a specific test for brucellosis but lacks sensitivity. It detects acute cases but misses chronic cases. IgM ELISA being more sensitive should be used for confirmation. Low ODs point to chronic brucellosis which was confirmed by IgG ELISA. Normal CRP levels in patients with PUO and chronic joint pains should point to brucellosis. KAP revealed that 25% to 50% of doctors considered Brucella in their differential diagnosis of acute and chronic fever respectively while 10% Orthopedics considered it in cases of arthralgia. ***Conclusion:*** Our results highlight the significance of *Brucella* as a cause of PUO and arthralgia. Brucellosis is an under-recognized but important cause of pyrexia of unknown origin and chronic joint pain. It should be actively suspected, diagnosed, and treated.

Keywords: ELISA, Pyrexia of Unknown Origin, Arthralgia, Brucellosis, Livestock.

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1. Introduction

Brucellosis is an emerging zoonotic disease with a worldwide distribution. It is known by various names like Malta fever, Mediterranean fever, undulant fever, or typhomalarial fever, names which are drawn from the geographical regions where these diseases are endemic, from the remittent character of fever or its resemblance with typhoid and malaria.[1] Despite significant disease morbidity and mortality, it remains a highly neglected tropical disease in our region. More than half a million new cases are reported each year. According to World Health Organization, this figure grossly underestimates the magnitude of the problem.[2,3] Brucellosis is a master at masquerading several infectious and non-infectious diseases, often leading to inappropriate diagnosis and management and under-reporting.

Brucellosis is an endemic disease in India with around 66% of the total population residing in 0.6 million villages.[4] Brucellosis in India was first reported in 1942.[5] Since then, cases have been reported from almost all its states[6] with seroprevalence ranging from 0.8-26%.[7] The disease is common among the young and middle-aged population in countries where it is endemic.[6] Taking cognizance of the significant morbidity and mortality associated with it, Brucellosis has been included in the top eleven priority zoonotic infections by the roadmap to Combat Zoonoses in India Initiative.[8]

A unique characteristic of the disease are its protean manifestations. While common symptoms are fever, chills, sweats, weakness, loss of weight, and abdominal pains, it is not rare for the disease to present as a respiratory illness, central nervous system infection, heart disease, urogenital infection, or chronic localized lesions. It is usually difficult to diagnose clinically as patients often present with pyrexia of unknown origin or joint pain only. The low index of suspicion, protean manifestations, and absence of cheap, sensitive, and specific diagnostic tests make this infection notoriously difficult to diagnose and is thus often treated inappropriately. The numbers reported in India is an underestimate due to the triad of lack of awareness of the disease, poor or absent community-based data, and non-availability of confirmatory laboratory tests. Diagnosis of Brucellosis entails blood cultures, demonstration of elevated antibodies or molecular techniques. In a resource poor country, where non-automated blood cultures have poor sensitivity, a positive diagnosis usually depends on a strong clinical suspicion and serological diagnosis.

The present study aimed to assess the seroprevalence of brucellosis in cases of pyrexia of unknown origin and/or in patients with joint pains with detailed documentation of clinical signs and symptoms. We compared the sensitivity and specificity of rapid, easy to use and cheap PUO screen latex agglutination test with an in-house validated IgM and IgG ELISA for Brucellosis. As C reactive protein (CRP) is a useful marker of inflammation, its levels were assessed in *Brucella*-positive individuals. To understand the current level of awareness about brucellosis in our center, we conducted a knowledge, attitude, and practices (KAP) survey amongst the clinicians. This survey was done concurrently with the assessment of seroprevalence to spread awareness about the prevalence of Brucellosis amongst the clinicians. This study was part of an ICMR sponsored STS project.

2. Material and Methods

This prospective, time bound pilot study was conducted in the Department of Microbiology in collaboration with the Departments of Medicine, Jawaharlal Nehru Medical College and Hospital, Pt Deen Dayal Upadhyay Hospital, Aligarh and Central Institute for Research on Goats (CIRG) Mathura. This study was conducted over two months from August to September 2016 after obtaining prior ethical approval from the Institutional Ethical Committee.

Study group

Consecutive patients presenting with PUO, arthralgia (with or without fever) and/or constitutional symptoms were recruited from two hospitals in Aligarh (J.N. Medical College Hospital and Pt. Deen Dayal Upadhyay Hospital). Apart from them, as an outreach effort, samples were also collected from adjoining rural areas from patients presenting with similar complaints to rural clinics. Written informed consent was obtained from each patient. PUO was defined as a fever greater than 38.3°C / 101°F on several occasions during a period longer than 3 weeks for which an etiology could not be established at the end of at least one-week's hospital stay. Detailed

clinical history and risk factors for brucellosis (close contact with animals, ingestion of raw milk, or work in an abattoir) based on predesigned proforma were elicited from all the patients.

Control group

The control group was drawn from the blood bank of J. N. Medical College. It consisted of 30 healthy people of comparable age: 22 (73.34%) were men and 8 (26.66%) women; their mean age was 37 years.

Sample collection

10-20 ml blood was collected aseptically from all the recruited patients for blood culture, serology, and routine investigations. Serum was separated by centrifugation, aliquoted, and stored at -20°C until further analysis was done.

Routine investigations

Complete hemogram, erythrocyte sedimentation rate, CRP, liver function tests, and renal function tests were performed.

Primary screening

Blood cultures were put up in all cases of PUO. Peripheral smear and/or QBC test was performed to exclude malaria and Widal was put up to rule out enteric fever. In cases negative for bacteremia/ septicemia, malaria and enteric fever, the presence of leptospiral infection was investigated by rapid agglutination test (Leptocheck, Tulip) as per the manufacturer's instructions. All *Leptospira*-negative samples were next processed for scrub typhus using the rapid test for scrub typhus, pyrexia of unknown origin (PUO) screen (Tulip).

Screening for Brucellosis

All PUO cases negative for enteric fever, malaria, leptospirosis, scrub typhus, and other bacterial infections, and all arthralgia cases were subjected to rapid test for brucellosis using PUO Screen (Tulip) which detects antibodies against *Brucella abortus* antigen. We compared its performance with two standardized tests developed by the Department of Microbiology, CIRG, Mathura. Indirect ELISA (iELISA) was used as a primary diagnostic assay in the current study. The cut-offs used as the diagnostic criteria in our study were 0.155 OD (optical density) as a positive control and 0.0465 OD as a negative control. 0.172 OD was considered as the lower limit for Brucella-positive cases. IgG ELISA was performed if iELISA ODs were low positives. For IgG estimation, BruLISA® was used. It was developed at the Indian Council of Agricultural Research- Central Institute for Research on Goats for detection of brucellosis. It is a validated test with a commercial license from National Research Development Corporation (NRDC), Ministry of Science and Technology, Govt. of India.

Assessment of CRP

CRP was assessed in all Brucella positive patients using the RHELAX-CRP Tulip diagnostic kit.

Statistical Analysis

SPSS IBM Statistics version 23 was used for the statistical analysis. As the study variables were categorized, Chi-square test or Fisher's exact test were used to find the association between brucellosis and other parameters. The difference was considered significant if $p<0.05$.

Knowledge, Attitude, and Practices (KAP)

Knowledge, attitude, and practices regarding Brucella among the clinicians were assessed as per a pre-designed questionnaire. This was carried out to generate awareness about Brucellosis among them.

3. Results

During this study, 419 patients (223, 139 and 57 patients presenting in JNMCH, Pt. DDU hospital and in rural clinic respectively) were screened for bacteremia, malaria, enteric fever, leptospirosis, and scrub typhus. From these a total of 92 patients, negative for sepsis, enteric fever, malaria, Leptospira, Rickettsia, and other bacterial infections were screened for Brucellosis. Female patients predominated, with the female-male ratio being 1: 0.877. Among these patients, 75 (81.5%) patients had a history of fever while 61 (66.3%) patients complained of joint aches. Duration of fever ranged from one month to six months in the majority (45.3%) of cases. Knee and vertebrae (including cervical region) were the most involved sites in patients with joint pains, followed by ankle and hip. Among these, 69 (72.82%) of the patients had a rural background (Figure 1). History of cattle rearing was elicited from 54 (58.6%).

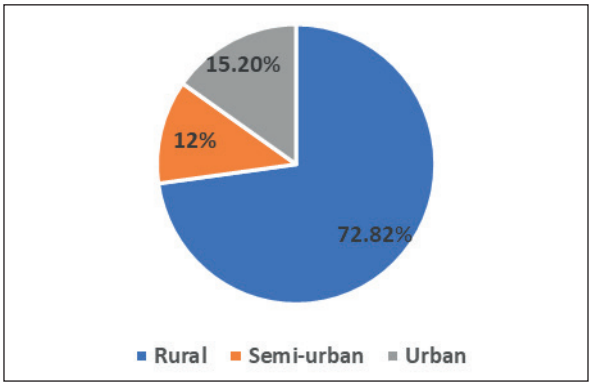


Figure 1: Distribution of patients based on their area of residence

Brucella serology

Overall prevalence of brucellosis in 419 cases of fever and arthralgia was 27 (6.4%) while it was 29.3% out of the 92 cases negative for all other common infections. 11 (11.96%) patients were positive for Brucellosis by PUO screen slide agglutination test (Figure 2) while 27 (29.3%) patients had IgM antibodies against *Brucella* as detected by iELISA. Eleven of these were strongly positive (> 0.350 O.D.) while 16 were positive but had lower O.Ds. (>0.172 and < 0.350). IgG ELISA was performed for the low positive IgM cases. These cases were strongly IgG positive and were diagnosed with chronic *Brucella*. PUO screen failed to antibodies in sera with low IgM ODs. This suggests that the PUO screen is effective in detecting acute cases of brucellosis and not chronic brucellosis. In our study, 11 (40.7%) patients were identified with acute brucellosis and 16 (59.3 %) had chronic brucellosis.

Comparison of PUO Screen test with iELISA for Brucellosis

Taking iELISA as the reference (gold standard) test, the 16 cases identified as negative by rapid PUO Screen were confirmed to be false negative and three cases were identified as false positive. The sensitivity and specificity of the rapid PUO screen test as calculated against ELISA was 29.62% and 95.5% respectively, with positive predictive value and negative predictive value being 72.2 % and 77.3 % respectively.

Clinical Profile of patients with Brucellosis

Twenty-seven (27/92; 29.3%) patients were positive for *Brucella* IgM antibodies of which 14 were males and 13 females. Male-female ratio was 1.07:1. Of these, eight patients belonged to urban, two to semi-urban, and seventeen to rural background. History of cattle and goat rearing was elicited from one urban, one semi-urban, and thirteen rural cases positive for *Brucella*.

The most common manifestations observed in the patients with brucellosis were fever (77.8%), arthralgia (77.8%) followed by fatigue (70.8%), pallor (66.1%), backache (53.8%), headache (59.2%), cough (33.3%), and weight loss/anorexia (33.3%). On comparing the clinical characteristics in *Brucella* positive and negative individuals, it was found that fever, fatigue, joint pain, and backache were significantly associated ($p < 0.05$) with Brucellosis infection.

On comparison of clinical features in various age groups, fever, arthralgia, fatigue, and headache

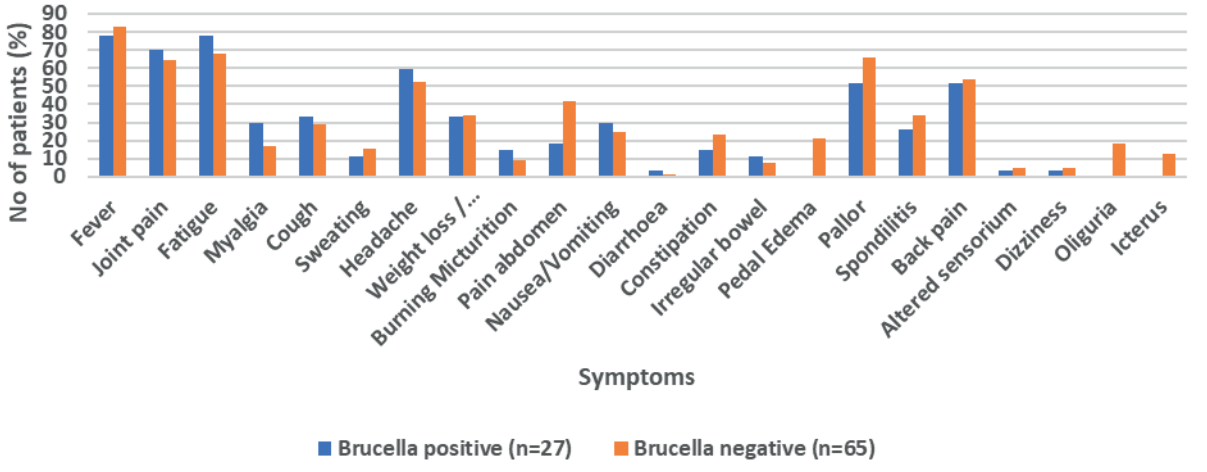
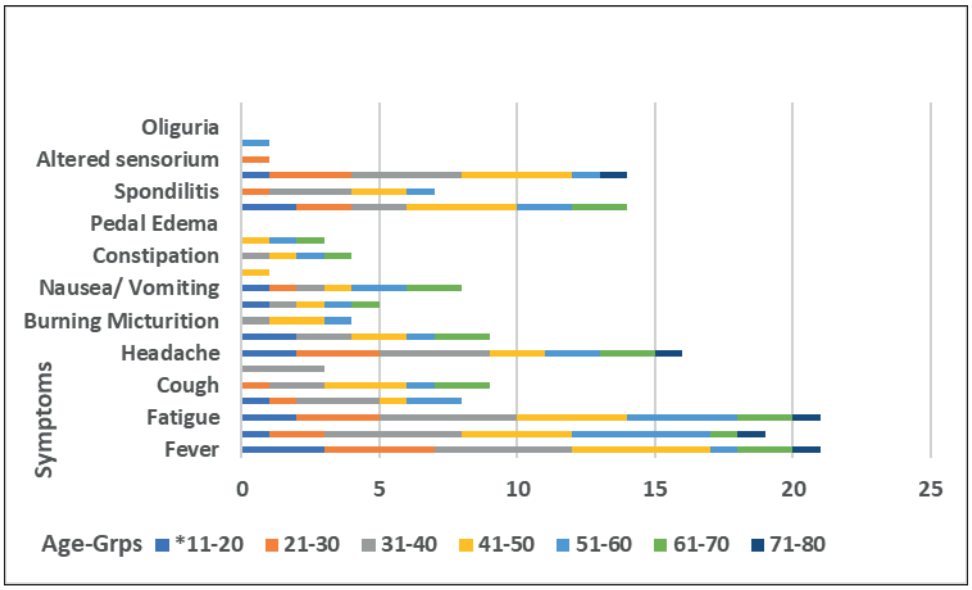


Figure 2: Comparison of protean clinical manifestations between *Brucella* positive and negative cases

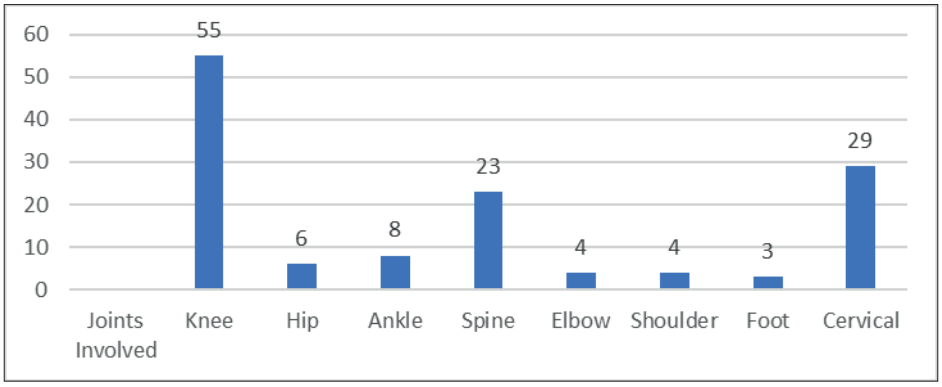
were present in all ages. Duration of fever in the *Brucella*-positive cases ranged from one week to six months. However, a large number (42.85%) of *Brucella* positive cases had fever which extended for one to two months (Figure 3).

Figure 3: Distribution of protean manifestations of Brucellosis in *Brucella* positive (n=27)



Many (21.7%) presented with spondylitis. Backache was seen in 56.5% of cases where the spinal area was the predominantly involved site. Knee and spine (including cervical) were the predominantly involved sites amongst the patients with complaints of joint pains, followed by ankle and hip. The knee joint was predominantly involved in the 41-50 year age group, followed by 51-60 years. Spine involvement was also predominant in 41-50 years of age, followed by 31-40-year age group. A similar trend was observed with cervical involvement which was maximum in 41-50 years of age followed by 31-40 and 51-60 years of age. (Figure 4)

Figure 4: Distribution of joints involved in patients



Assessment of CRP among the study population

CRP was overall positive in patients presenting chiefly with complaints of fever (88.9%), fatigue (72.2%), pallor (72.2%), headache (61.1%), and joint pains (50.0%). However, it did not prove to be a useful biomarker of *Brucella* infection in our study with only 5 (18.5%) cases being CRP reactive. However, these 5 cases were all diagnosed with acute Brucellosis. These findings suggest an interesting corollary: normal CRP levels point towards Brucellosis.

KAP Analysis

In fevers of < 1month, the top two differential

diagnosis (D/D) by 100% -90% of doctors were malaria and viral fever, followed by Leptospira and Rickettsia. Only 25% considered Brucellosis in their D/D. It was good to note that due to our prior studies and KAP, leptospirosis and Rickettsia had started featuring in their differential diagnosis. In fevers of > 1month, the top two differential diagnosis by all (100%) and 70% of doctors were tuberculosis and HIV respectively. However, it was heartening to observe that 50% did consider brucellosis. In contrast, only 10% considered brucellosis in their D/D in patients presenting with joint pains (Table 1).

Table 1. Assessment of Doctor's knowledge and awareness of Brucella as a cause of fever of less and more than one month and joint pain

Diagnosis	Doctor's response n=20 (%)
Differential diagnosis for fever of <1month	
1.Malaria	20 (100%)
2.Viral fever (Dengue)	18 (90%)
3.Leptospira	14 (70%)
4.Rickettsia	10 (50%)
5.Brucellosis	5 (25%)
Differential diagnosis for fever of >1month.	
1.Tuberculosis	20 (100%)
2.HIV	14 (70%)
3.Brucellosis	10 (50%)
4.Rickettsia	10 (50%)
Differential diagnosis for joint pains.	
1.Osteoporosis	20 (100%)
2.Osteoarthritis	20 (100%)
3.Vitamin D deficiency	16 (80%)
4.Rheumatoid arthritis	11 (55%)
5.Spondilitis	10 (50%)
6.Pott's spine	5 (25%)
7.Brucellosis	2 (10%)

Analysis of the KAP (knowledge, attitude, and practices) regarding *Brucella* revealed that out of 20 doctors assessed, only 5 (25%) of doctors considered *Brucella* in their differential diagnosis (D.D.) for fever of less than 1 month, 10 (50%) doctors considered it for fever of more than one month, and only 2 (10%) doctors considered it in patients presenting with arthralgia.

4. Discussion

The protean manifestations of brucellosis, low index of suspicion, and absence of cheap, sensitive, and specific diagnostic tests make this infection notoriously difficult to diagnose and thus often erroneously treated. The disease often leads to a partial or complete loss of physical efficiency and loss of valuable productive days.[11,12] If correctly diagnosed, treatment of Brucellosis is easy and affordable, and it responds dramatically to appropriate antimicrobials.

In this study, we report a high prevalence of (29.3%) Brucellosis in our region. Other studies have reported a prevalence ranging from 0.8-26% from different parts of the country.[7]

Brucellosis certainly poses a serious threat to public health. It needs to be differentiated from other febrile illnesses like enteric fever, malaria, tuberculosis, leptospirosis, infectious mononucleosis, etc. Its protean clinical manifestations like intermittent to remittent fever, joint aches, malaise, fatigue, headache, backache, profuse nocturnal sweating require a high index of suspicion to initiate appropriate investigations, early diagnosis, and prompt management of the disease. [13,14]

Apart from fever (77.8%) and arthralgia (70.4%), the five most common clinical features observed in Brucellosis patients in our study were fatigue, pallor, backache, headache, and cough. Duration of fever in Brucellosis cases ranged from one week to six months, of which a large number (42.85%) had fever for one to two months. Our study highlights that pyrexia of longer duration should alert a doctor towards not only tuberculosis and other chronic illnesses but towards Brucellosis also. Roushan *et al.*[15] reported that fever and arthralgia were the most common symptoms in their study. Osteoarticular involvement has been reported in 20-60% of cases of brucellosis.[16] In our study it was observed that joint aches were strongly associated with *Brucella* infection. A significant majority (69.6%) of *Brucella*-positive patients complained of arthralgia and many (21.7%) presented with spondylitis. Backache was seen in 56.5% of cases with the spinal area being the predominant site. Our findings suggest that not only physicians and pediatricians but orthopedics should also maintain a strong index of suspicion for Brucellosis.

Most of the patients in our study population had a rural background. Many were agriculturists and were involved in livestock rearing placing them at a higher risk of getting infected. History of livestock rearing was elicited from 15 (55.55%) *Brucella* positive cases, among which thirteen were from rural areas while one each belonged to an urban and a semi-urban area.

Serological tests measuring specific antibodies to *Brucella* lipopolysaccharide are quite sensitive and specific and are of great importance in the initial detection of disease.[17] They should become the mainstay in laboratory diagnosis. In developing countries like India, the prohibitive cost of conducting high-end investigations like PCR on the one hand and time-consuming technique of culturing the bacteria though accurate, may not be the most feasible diagnostic aids for Brucellosis.

We assessed a slide agglutination test-PUO Screen (Tulip Diagnostics) which was compared with IgM and IgG ELISA. Both these tests are specific for Brucellosis. IgM iELISA detected 29.3% positive cases while the positivity rate by slide agglutination was much lower at 11.96%. The sensitivity of the PUO screen was poor at 29.62% but specificity was high at 95.5%. The sera with higher ODs in IgM iELISA (11/27, 40.7%) were largely detected by PUO screen while lower ODs were not detected. IgG iELISA was however strongly positive in those sera which had low ODs in IgM iELISA and they were subsequently identified as chronic Brucellosis. Thus, in our study, 40.7% had acute brucellosis and 59.3 % had chronic brucellosis. Hence screening of suspected cases can be performed by slide agglutination. Since it has high specificity, it can be considered a good diagnostic tool for the detection of acute brucellosis. All negatives by PUO Screen should be tested by both IgM ELISA and IgG ELISA to distinguish between acute and chronic cases. In a study conducted in 2011, the prevalence of Brucellosis in South Karnataka was found to be 2.14% and North Karnataka 0.92%.[3] The seropositivity in that study was detected only by indirect ELISA by using SLPS of *Brucella abortus*-99. [17] A study from North India conducted over a decade in patients of PUO reported a fluctuating seroprevalence ranging from 4% to 18% using serum agglutination test (SAT) to detect *Brucella* agglutinins.[12]

In this study, we assessed the utility of CRP as an indirect marker of Brucellosis. Overall CRP did not prove to be an efficient biomarker for detecting *Brucella*, although it was high in acute cases and low in chronic cases. Thus, it can be used to differentiate between acute and chronic brucellosis. Others have reported high levels of CRP in their studies in acute Brucellosis.[18,19,20] CRP being an acute-phase reactant is raised in acute bacterial infections.[21] Thus, low CRP levels may point towards Brucellosis and especially so towards chronic brucellosis.

KAP analysis revealed poor awareness amongst the doctors regarding brucellosis. In fevers of < 1 month, the most common differential diagnosis were malaria and viral fever, followed by Leptospira and Rickettsia. Only 25% considered Brucellosis in their D/D. It was good to note that due to our prior studies and KAP, leptospirosis and Rickettsia had started featuring in their differential diagnosis.[22] In fevers of > 1month, the top two differential diagnoses were tuberculosis and HIV although it was heartening to note that 50% did consider brucellosis. In contrast, only 10% considered brucellosis in their D/D in patients presenting with joint pains.

Thus, it is imperative to enhance awareness among doctors about the prevalence of this disease and at the same time introducing appropriate diagnostic tests. Microbiologists play a pioneering role in bringing neglected tropical diseases to center-stage and must spearhead early diagnosis and prompt treatment of such diseases. They need to vet different diagnostic tests and adopt appropriate validated tests which are cheap but at the same time sensitive and specific so that timely and accurate diagnosis can be made.

5. Conclusion

To conclude, slide agglutination test-PUO screen can serve as an initial but not the sole diagnostic method for *Brucella* infection as it has low sensitivity but high specificity. Thus, it is possible for most microbiology laboratories to start screening for Brucellosis by PUO screen, assess the burden in their area, and later add more sensitive tests like IgM/IgG iELISA depending upon the seroprevalence and facilities available. Our results show that Brucellosis with a prevalence of 29.3% is an important cause of PUO. This was a time bound pilot study. Active large-scale surveillance

of *Brucella* infection is essential to assess the exact magnitude and distribution of the disease.

Conflict of interest: The authors have no conflicts of interest.

Funding Statement: None declared.

Ethical Approval Issue: This study was conducted after obtaining permission from institutional ethics committee of J.N. Medical College.

Authors' contribution: All authors in the study read the manuscript and made great input.

Supplementary file:

Sample Collection and Transportation:

5 ml blood was obtained by venupuncture taking all aseptic precautions, in plain vial. Preparation of site:

- Vein must be chosen before blood is withdrawn.
- If a patient has an existing IV line, blood should be withdrawn below the existing line.
- Once vein is selected the skin site is defatted with 70% isopropyl alcohol in a circle approximately 5 cm in diameter rubbing vigorously. Allow to air dry.
- Starting in the centre of the circle, apply 2% tincture of iodine in ever widening circles until the entire circle has been saturated with iodine. Allow the iodine to dry on the skin for at least one minute.
- If the site must be touched by the phlebotomist after preparation, gloved fingers used for palpation should be disinfected in a similar fashion.
- Insert needle into the vein and withdraw blood.
- After needle has been removed the site should be cleansed with 70% alcohol again.

Serum was separated from sample in plain vial by centrifugation and stored in SV4 vials at -20ºC or -70ºC for months.

Preparation of iELISA:

iELISA protocol as per the method of Engvall and Pearlman (1971) was employed in this study with slight modifications. The iELISA technique was already developed and standardized in the CIRG laboratory using soluble *Brucella melitensis* protoplasmic antigen. The polypropylene ELISA plates were coated with 500 ng *Brucella melitensis* (Biovar 3) smooth LPS antigen per well using a carbonate-bicarbonate buffer [9, 10]. The pre-coated plates were stored at 4°C until further use. The plates are washed three times with 250µl of 1X PBS-Tween buffer (PBST with 0.05% Tween20), followed by blocking with 3% Bovine serum albumin (BSA) in 1X PBS and plates were incubated at 37°C for one hour followed by washing with PBST. The serum samples were diluted at the rate of 1:50 using serum dilution buffer, prepared using 1% BSA in 1X PBST and added to the wells, and incubated for two hours, then washed. Rabbit anti-goat IgG HRP conjugate at 1:3000 dilution in 1xPBS was added and incubated at 37°C for 30 min. For IgM iELISA, the conjugate used is rabbit anti-goat IgM HRP at a dilution of 1:2000, with all other steps essentially the same as described here. Substrate buffer (prepared using 2.1% citric acid and 3.56% Na_2HPO_4 freshly dissolved in triple-distilled water with 1mg OPD tablet and 100µl H_2O_2) was added at the rate of 100µl per well followed by an incubation of 15 minutes. The optical density was measured at 450nm using the microplate reader MultiSkan Go™ (Thermo scientific, USA). The duplicate OD values were used to derive the mean and standard deviation. The results were interpreted as per the negative cut off value which was calculated using the formula: 'Mean OD of negative control + 2× Standard Deviation'.

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